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Pharmacological Inhibition of Voltage-gated Ca^{2+} Channels for Chronic Pain Relief

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Abstract: Chronic pain is a major therapeutic problem as the current treatment options are unsatisfactory with low efficacy and deleterious side effects. Voltage-gated Ca^{2+} channels (VGCCs), which are multi-complex proteins consisting of α_1 , β , γ , and $\alpha_2\delta$ subunits, play an important role in pain signaling. These channels are involved in neurogenic inflammation, excitability, and neurotransmitter release in nociceptors. It has been previously shown that N-type VGCCs ($\text{Ca}_v2.2$) are a major pain target. U.S. FDA approval of three $\text{Ca}_v2.2$ antagonists, gabapentin, pregabalin, and ziconotide, for chronic pain underlies the importance of this channel subtype. Also, there has been increasing evidence that L-type ($\text{Ca}_v1.2$) or T-type ($\text{Ca}_v3.2$) VGCCs may be involved in pain signaling and chronic pain. In order to develop novel pain therapeutics and to understand the role of VGCC subtypes, discovering subtype selective VGCC inhibitors or methods that selectively target the inhibitor into nociceptors would be essential. This review describes the various VGCC subtype inhibitors and the potential of utilizing VGCC subtypes as targets of chronic pain. Development of VGCC subtype inhibitors and targeting them into nociceptors will contribute to a better understanding of the roles of VGCC subtypes in pain at a spinal level as well as development of a novel class of analgesics for chronic pain.

Keywords: $\alpha_2\delta$ subunit, Inhibition, L-type Ca^{2+} channels, N-type Ca^{2+} channels, Pain, Targeting, T-type Ca^{2+} channels, Voltage-gated Ca^{2+} channels.

1. INTRODUCTION

Pain is the somatosensory detection of noxious stimuli, helping our body be aware of damaging stimuli. These signals are transmitted into the central nervous system through a subset of peripheral nerve fibers, which are called nociceptors. These consist of thinly myelinated A δ and unmyelinated C fiber primary sensory neurons [1]. These neurons are pseudobipolar as they have two peripheral and central branches arising from a single axon coming from the cell body. These cell bodies are clustered in dorsal root ganglia (DRG). The sizes of the cell bodies of nociceptors range from small (100-400 μm^2) to medium (400-1200 μm^2). Generally, large DRG cells are not considered to be nociceptive neurons. The presynaptic terminals, which are the central branches of nociceptors, form synapses with second order neurons in specific locations of the spinal cord. For example, A δ fibers terminate in lamina I and V, while C fibers project to Lamina I and II. Neurotransmitter release in the synapse is the primary mode of transmission to the central nervous system from nociceptors. Substance P, CGRP, and glutamate are representative examples of these neurotransmitters. The peripheral nerve endings innervate the skin and viscera that receive either the high threshold internal (inflammatory soups) or external (heat, acid, and

pressure) signals. These nerve endings also release substance P and CGRP, which may contribute to neurogenic inflammation [2].

Nociceptors are specialized high-threshold neurons that respond to noxious stimuli. The peripheral nerve endings of nociceptors express specialized high threshold receptors such as ligand gated ion channels (TRP channels, P2X channels, and ASIC channels, etc) and G-protein coupled receptors (Mas-related G-protein class, etc). These proteins convert high threshold external or internal stimuli into electrical signaling such as action potentials, which are propagated through primary sensory neurons. Here, voltage-gated Na^+ channels ($\text{Na}_v1.3$, $\text{Na}_v1.7$, and $\text{Na}_v1.8$), as relay stations, are in charge of this action potential propagation. As a final step in the peripheral nervous system, the electrical signaling ends in presynaptic terminals and transmits to second order neurons in the spinal cord. In the presynaptic terminals, neuronal voltage-gated Ca^{2+} channels (VGCCs, in particular, $\text{Ca}_v2.2$) play an important role in pain neurotransmitter release such as CGRP, Substance P, and glutamate. Taken together, this series of events (transduction, propagation, and transmission) in peripheral nociceptors comprise pain signaling.

While the pain signaling works as a physiological defense system to protect ourselves from harmful conditions, persistent tissue and nerve injury cause chronic inflammatory and neuropathic pain, which comprise major medical problems. Inflammatory pain is caused by tissue injury, which release inflammatory mediators containing cytokines

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and neuropeptides. Neuropathic pain is caused by neural damage by HIV infection, cancer, mechanical trauma, and spinal cord injury. Dysfunctional pain is defined by pain with no identifiable sources. Fibromyalgia, irritable bowel syndrome, interstitial cystitis, erythromelalgia, paroxysmal extreme pain disorder, and complex regional pain syndrome (CRPS) are examples of neuropathic or dysfunctional pain. Nociceptive or inflammatory pain can be called adaptive because the pain usually disappears after avoidance of stimuli or resolution of injury. The neuropathic pain and dysfunctional pain are maladaptive, in which pain occurs without identifiable and detectable stimuli (spontaneous pain) and also with hyperalgesia and allodynia [3]. These symptoms are a result of the neuron's increased sensitivity to painful- and normal-threshold stimuli, and hyperexcitability. Modulations of ion channels and GPCRs by chronic pathological pain are involved in high sensitivity or hyperexcitability.

A significant amount of progress in understanding chronic pain mechanisms has been achieved, although it is still not complete due to its complexity and multiple mechanisms. Many proteins have been suggested as targets for pain therapeutics; however, actual therapeutic targets are few in number. The nociceptive and inflammatory pain currently are mainly addressed with NSAIDs and opioids that inhibit COX enzymes and activate opioids receptors, respectively. However, current therapeutics for chronic and maladaptive pain are not satisfactory. The major problems of current chronic pain medicines are side effects and low efficacy [4]. For example, opioids and NSAIDs (e.g. codeine and ibuprofen) have several side effects such as tolerance and gastric problems, respectively. More importantly, they are not considered effective especially against neuropathic chronic pain. Number needed to treat (NNT; an epidemiological measure of effectiveness) of these drugs is around 4 (NNT, 1 is ideal and 2.5 is satisfactory). Other than these medications, antidepressants, antiepileptics, topical capsaicin, and local anesthetics have been used for chronic neuropathic pain, but they still have the same limitations as opioids and NSAIDs [5]. With an unmet need for pain therapeutics, it is important to pursue the development of novel therapeutics with high efficacy and low side effects.

Among the targets of chronic pain, inhibiting VGCCs in nociceptors is an effective way for reducing pain. As mentioned above, VGCCs are directly involved in neurotransmitter release at synapses in the spinal cord, as well as neurogenic inflammation in the peripheral nerve endings. This means that VGCCs for neurons of the peripheral nerve system are end points for both transmitting pain signals into central nervous system and for evoking neurogenic pain in the periphery. In addition, many endogenous factors up-regulated by neuropathic pain modulate VGCCs to induce hypersensitivity [6]. Currently, three drugs targeting VGCCs are commercially available for neuropathic pain medication; pregabalin, gabapentin, and ziconotide. However, pregabalin and gabapentin have serious side effects and low efficacy, and also their role in inhibiting VGCCs is controversial. Ziconotide still has severe side effects and requires intrathecal administration through a catheter and pump. On the positive side, it doesn't

lead to tolerance and dependence and is more effective for refractory pain than even opioids [7]. In this regard, discovering more selective and potent drugs for VGCCs and targeting them into nociceptors is beneficial for chronic pain therapeutics. The following will describe VGCC subtypes as targets of chronic pain, VGCC subtype inhibitors, and the possibility of therapeutic application and strategies based on the characteristics of VGCCs.

2. VOLTAGE-GATED Ca^{2+} CHANNELS

Calcium ions (Ca^{2+}) exist at a higher level of concentration outside the cell membrane (~ 2 mM) than inside (~ 50 – 100 nM). The Ca^{2+} gradient is maintained until the activation of VGCCs by membrane depolarization, which is evoked by activation of ligand gated ion channels or GPCRs. The resulting Ca^{2+} influx (10 to 100 times increase of intracellular Ca^{2+}) plays a role in muscle contraction, hormone secretion, synaptic transmission, enzyme activity, and gene expression in different cell types such as muscle, endocrine cells, and neurons [8, 9].

The functional VGCCs are a protein complex of $\alpha 1$, $\alpha 2\delta$, β , and γ subunits. The $\alpha 1$ subunits are the key molecule of the channel complex because it is capable of Ca^{2+} conduction, or generating Ca^{2+} influx. The $\alpha 2\delta$, β , and γ subunits are auxiliary. They increase the expression of $\alpha 1$ subunits in the plasma membrane as well as modulate the function of $\alpha 1$ subunits, resulting in functional diversity in different cell types. The $\alpha 2\delta$ subunits increase maximum currents by trafficking of the $\alpha 1$ subunits. Also, they increase the inactivation rate as well as shift steady state inactivation into hyperpolarization. The β subunits located in the cytosol also increase maximum currents by enhancing trafficking of $\alpha 1$ subunits, shifting activation to the left, and increasing open probability. However, the role of γ subunits as auxiliary subunits is controversial. Rather, they are more important in modulating AMPA receptors. A recent review described other functions of auxiliary subunits of VGCCs [10].

The $\alpha 1$ subunit consists of ~ 2000 amino acids and four repeated domains, each of which is homologous with voltage-gated potassium channels (VGPCs). These domains are connected with three intracellular loops. In each domain, first four transmembrane segments (S1–S4) form the voltage sensing domain, while S5, P-loop, and S6 form the pore domain (Fig. 1). Based on sequence differences, the mammalian $\alpha 1$ subunits are currently classified into 10 subtypes. These subtypes have different biophysical properties (activation, inactivation, conductance, and deactivation), expression patterns, and pharmacology (Table 1).

L-type VGCCs, first characterized from cardiac muscle, include four genes ($\text{Ca}_v1.1$ – 1.4). These channels are activated at high voltage, thus classified as HVA (high voltage activated) Ca^{2+} channels. Their half-maximal activations are around -20 to 0 mV, except for $\text{Ca}_v1.3$, which is activated by low voltages (-30 ~ -20 mV). These channels are mainly expressed in cardiac and skeletal muscle cells and responsible for muscle contraction, hormonal secretion, and gene transcription. Dihydropyridine (DHP) antagonized L-type VGCCs and had been useful for isolating and characterizing these channels. Also, phenylalkylamine

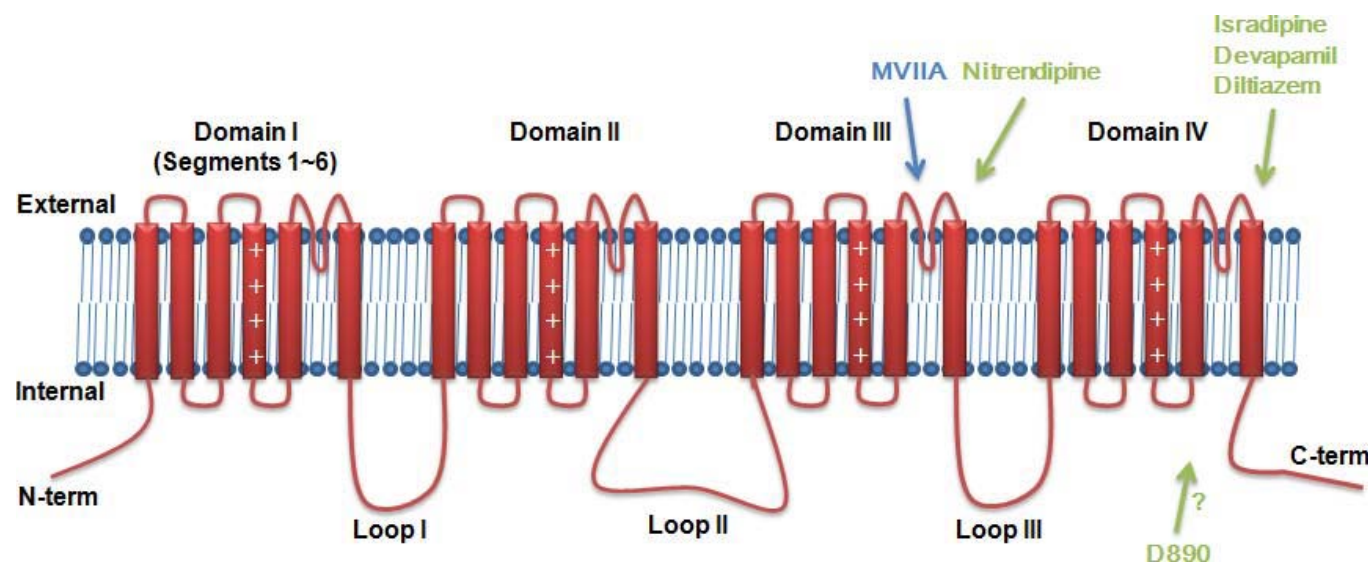


Fig. (1). Primary structure of $\alpha 1$ subunits of VGCCs.

Table 1. Classification of Voltage-gated Ca^{2+} Channels

	Current Name	Channel Name	Gene Name	Half Activation Voltage (Reference [23])
High-voltage activated (HVA)	L-type	$\text{Ca}_v1.1$	CACNA1S	8~14mV
		$\text{Ca}_v1.2$	CACNA1C	-4~-18.8mV
		$\text{Ca}_v1.3$	CACNA1D	-15~-37mV
		$\text{Ca}_v1.4$	CACNA1F	-2.5~-12mV
	P/Q-type	$\text{Ca}_v2.1$	CACNA1A	-5~10mV
	N-type	$\text{Ca}_v2.2$	CACNA1B	7.8~9.7mV
	R-type	$\text{Ca}_v2.3$	CACNA1E	-29.1~3.5mV
Low-voltage activated (LVA)	T-type	$\text{Ca}_v3.1$	CACNA1G	-46mV
		$\text{Ca}_v3.2$	CACNA1H	-46mV
		$\text{Ca}_v3.3$	CACNA1I	-44mV

(PAA) and benzothiazepine (BTZ) have antagonizing effects against the channels.

P/Q-type VGCCs ($\text{Ca}_v2.1$) were found to be expressed in purkinje cells and cerebellar granule neurons, and N-type VGCCs ($\text{Ca}_v2.2$) were characterized from the brain [11]. These channels are expressed in presynaptic nerve terminals and play an important role in neurotransmitter release. These two types have been known to play a complementary role. These channels are antagonized by peptide neurotoxins ω -Agatoxin IVA and ω -conotoxin MVIIA, respectively. Residual current of R-type VGCCs ($\text{Ca}_v2.3$) still remains after blocking L-, N-, P/Q-type VGCC subtypes. Another peptide neurotoxin SNS-482, isolated from the tarantula *Hysteroecrates gigas*, inhibits R-type VGCCs. The expression of P/Q-type, N-type, and R-type Ca_v channels are restricted to neurons. R-type VGCCs as well as L-type VGCCs ($\text{Ca}_v1.3$

only) are activated at rather lower voltage than other channels, but still are classified as HVA channels.

The other class of VGCCs ($\text{Ca}_v3.1\sim3.3$) are LVA (low voltage activated) T-type VGCCs. These channels are activated with half voltage activation of -46 ~ -44mV, thus open at resting membrane potentials. These channels regulate pacemaking, subthreshold activation, and repetitive firing in cardiac or neuronal cells. Interestingly, T-type VGCCs are not modulated by auxiliary subunits such as $\alpha 2\delta$ and β subunits [12, 13].

In addition to subunit associations and subtype differences, alternative splicing gives more diversity in the function of VGCCs. For example, N-type VGCCs have a number of splice variants, which depend on developmental stage and cell types [14, 15]. The splicing events are regulated by a neuronal splicing factor such as Nova [16].

For example, Nova-2 increases inclusion of e24a and decreases inclusion of e31a of N-type VGCCs in brain [17]. These variants showed distinct gating properties such as channel activation, inactivation and drug responsiveness. It has been shown that e37a of N-type VGCCs are expressed in capsaicin sensitive neurons, which are involved in pain behaviors. Further, in order to see the specific role of e37a of N-type VGCCs, e37a was replaced with e37b in mice. These mice showed no differences in pain behaviors, but less responsiveness to morphine. This fact suggested that splice variants can affect the pharmacology of N-type VGCCs and alternative splicing by splicing factors are important for pain signaling [18-20].

In generating action potentials, voltage-gated Na^+ channels (VGSCs) and voltage-gated K^+ channels (VGPCs) are involved in rising and falling phase. Thus, VGSC blocker abolishes action potentials. However, in contrast to VGSC blockers, VGCC blockers have shown small effects on action potentials. For example, L-type VGCC blockers abolish contractile force without a major change in the action potentials. Generally, VGCC blockers broaden the falling phase of action potentials [21]. However, it has been argued that less contribution of L-type VGCC currents in action potentials result from slow and use-dependent inhibition of L-type VGCC blockers [22]. Notably, T-type VGCCs are activated at near resting membrane potentials; therefore, it will amplify weak signals and cause low-amplitude intrinsic neuronal oscillations. T-type VGCC knockout ($\text{Ca}_v3.1^{-/-}$) shows reduced burst firing in thalamocortical neurons, suggesting its contribution to repetitive firing of action potentials. Two VGCC subtypes, $\text{Ca}_v1.3$ and $\text{Ca}_v2.3$, are activated at relatively low voltage so that they may have distinct roles with other HVA Ca^{2+} channels such as spontaneous firing or rhythmic burst discharges [23, 24]. These diverse characteristics of each subtype orchestrate neuronal spikes in sensory nervous system.

3. VGCC SUBTYPES AND PAIN

The role and importance of each VGCC subtype in pain signaling would be firstly glimpsed by where they are expressed in pain processing in the DRG and spinal cord. Further, the effect of genetic ablation of VGCC subtypes in mice on pain behaviors gives invaluable information about their function in pain. Also, their roles in chronic pain mechanisms, which are related to peripheral and central sensitization, would be determined by up- or down-regulation by neuropathic and inflammatory pain. It has been shown that various subtypes of VGCCs are expressed in dorsal root ganglia (DRG) according to their sizes [25]. In situ hybridization using RNA probes showed that all subtypes of VGCCs are expressed in rat DRG neurons except $\text{Ca}_v1.4$ and $\text{Ca}_v3.1$. It also has been shown that $\alpha\delta 1$ and $\alpha\delta 2$ subtypes are expressed in small and medium neurons (possibly nociceptors), while $\alpha\delta 3$ subtype is expressed in large neurons [26]. VGCC subtypes are also expressed in the spinal cord, suggesting critical roles in pain signaling through second order neurons in the spinal cord [27, 28]. In presynaptic nerve terminal of DRG and postsynaptic cells in spinal cord, VGCCs play different roles. VGCC subtypes in presynaptic terminals are involved in

neurotransmitter release and excitability; those in the spinal cord are involved in excitability and central sensitization [29]. In peripheral nerve endings, it has been suggested that VGCC subtypes play an important role in peptide release, which might be involved in neurogenic inflammation [2]. Several reviews have already described the importance of VGCC subtypes in terms of pain signaling [30, 31]; this review focuses on the particular roles of VGCC subtypes based on recent evidence on their localization, genetic ablation, and regulation upon chronic pain.

3.1. L-type Ca^{2+} Channels

L-type VGCCs are broadly expressed in skeletal and cardiac muscle, neurons, auditory hair cells, pancreatic cells, and retina [32]. Several lines of evidence have shown that DRG and spinal cord neurons express $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ [33, 34]. Electrophysiology in DRG showed that L-type VGCCs are present largely in small and large neurons [25]. Whether these channels are regulated in chronic pain state is not conclusive. One study using RT-PCR showed that L-type VGCCs are down-regulated in DRG upon chronic constriction injury (CCI) and sciatic nerve axotomy in rats [35]. They suggested that decreases of $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ in DRG contribute to hyperexcitability of neuropathic pain by modulating Ca^{2+} -dependent inactivation or facilitation as a negative feedback [36]. Inversely, it has been shown that $\text{Ca}_v1.2$ is up-regulated in the spinal cord in the sciatic nerve ligation (SNL) model. In this study, it has also been shown that knockdown of $\text{Ca}_v1.2$ using siRNA and peptide nucleic acids (PNA) reversed the neuropathic pain, while that of $\text{Ca}_v1.3$ did not [37]. This fact suggests that spinal $\text{Ca}_v1.2$ is important in pain signaling. It was supported by miR-103 decreasing translational levels of $\text{Ca}_v1.2$. Neuropathic pain decreases miR-103 and thus increases $\text{Ca}_v1.2$, resulting in hyperexcitability and allodynia [38]. $\text{Ca}_v1.3$ rather is involved in other sensory modalities or motor neurons according to its expression in lamina IX in spinal cord [39]. Knockout mice of $\text{Ca}_v1.3$ did not display atypical pain behaviors. The lack of effects in $\text{Ca}_v1.3^{-/-}$ mice might result from compensation effects, but the fact that RNAi knockdown of $\text{Ca}_v1.3$ did not affect pain behavior supports diminished role of $\text{Ca}_v1.3$ to pain signaling [40, 34]. Overall, although $\text{Ca}_v1.3$ is expressed in the spinal cord and brain stem, it seems to have fewer effects on pain signaling. However, it could not be ruled out because rather low voltage activation of $\text{Ca}_v1.3$ might contribute to spontaneous neuronal firing similar with T-type VGCCs. Another interesting aspect of L-type VGCCs in pain processing is their expression in peripheral nerve endings. Ca^{2+} influxes in corneal nerve terminals branching A δ - and C-fibers are inhibited by nifedipine and diltiazem [2]. This result suggests that L-type VGCCs also are involved in neuropeptide release in peripheral nerve endings, which contribute to neurogenic inflammation.

3.2. P/Q-type Ca^{2+} Channels

P/Q-type VGCCs are more centrally expressed than peripherally, thus being more associated with migraine. Missense mutations of P/Q-type VGCCs cause familial hemiplegic migraine (FHM) [41]. It has been known that neuronal P/Q-type VGCCs are complementary to N-type

VGCCs in the central nervous system. For example, these two channels mediate neurotransmitter release in distinct hippocampal inhibitory neurons [42]. Also, in DRG neurons, $\text{Ca}_v2.1$ is not localized with substance P or CGRP, which showed similar localization with $\text{Ca}_v2.2$. ω -agatoxin IVA, a P/Q-type VGCC blocker, did not affect the release of substance P or CGRP [27, 43]. In the spinal cord, they are located in presynaptic nerve terminals of lamina II-VI, while $\text{Ca}_v2.2$ is in the presynapse of lamina I and II. Therefore, it is likely that P/Q-type VGCCs are more involved in polysynaptic transmission rather than monosynaptic transmission between nociceptors and lamina I and II [29]. ω -agatoxin TK, a P/Q-type VGCC blocker, had no effect on postsynaptic Ca^{2+} influx in lamina I [29]. However, we cannot rule out the possibility of relevance to chronic pain, because a recent report showed that induction of spinal LTP, a marker of chronic pain, was strongly suppressed with ω -agatoxin IVA [44].

In accordance with its expression, the effects of $\text{Ca}_v2.1$ in pain signaling are rather complicated. In SNL neuropathic pain model, ω -agatoxin IVA has little effect on pain behaviors [45-47]. In knockout mice for $\text{Ca}_v2.1$, mice show enhanced sensitivity to thermal stimuli. Also, two P/Q-type VGCC mutant mice *Leaner* mice (*tgla/tgla*) and *Roller* mice (*tgrol/tgrol*) have been examined for pain behaviors. *Leaner* mice (*tgla/tgla*) showed reduced mechanical pain but enhanced heat pain like knockout mice. However, *Roller* mice (*tgrol/tgrol*) showed reduced sensitivity to heat pain in a gene concentration-dependent manner [48-50]. These facts suggest that the role of P/Q-type VGCCs in pain is complicated and is involved in different sensory modalities compared to N-type VGCCs. Therefore, the role of P/Q-type VGCCs in pain should be interpreted in specific contexts, as another review suggested [30].

3.3. N-type Ca^{2+} Channels

$\text{Ca}_v2.2$ is expressed in the central and peripheral nervous systems including the brain, spinal cord, and primary sensory neurons [51, 27]. It has been reported that $\text{Ca}_v2.2$ and substance P expression overlapped in DRG and the spinal cord. In accordance with their location, $\text{Ca}_v2.2$ antagonists ω -conotoxins MVIIA and GVIA block neurotransmitter release in the spinal cord [43, 52-54]. Autoradiography of ω -conotoxins also show that $\text{Ca}_v2.2$ is rich in laminae I and II [55, 56]. However, contrary to suggested specific localization of $\text{Ca}_v2.2$ with substance P, electrophysiology in DRG shows that N-type VGCC current are present evenly regardless of the size [25, 57]. In terms of regulation, it has been shown that the expressions of $\text{Ca}_v2.2$ are modulated by inflammatory and neuropathic pain. For example, inflammation by intraplantar CFA increases N-type VGCC protein expression but not mRNA [58]. CCI model, a neuropathic pain model, showed increased immunoreactivity against $\text{Ca}_v2.2$ in lamina II in spinal dorsal horn [59]. However, partial sciatic nerve injury (PSNL) did not regulate $\text{Ca}_v2.2$ but rather up-regulate the $\alpha2\delta$ subunit [60]. Inflammation by intraplantar CFA reduced the contribution of N-type VGCCs in NK1 positive neuron in lamina I [61]. Therefore, up-regulation of $\alpha1$ subunit of $\text{Ca}_v2.2$ by inflammation and nerve injury is ambiguous. Rather,

increase of $\text{Ca}_v2.2$ depends on $\alpha2\delta$ subunit, which traffic $\text{Ca}_v2.2$ into cell membranes because mRNA of $\text{Ca}_v2.2$ has not been changed upon inflammatory pain [58]. Also, it is possible that $\alpha2\delta$ subunit cause synaptogenesis so that increase of synaptic area affects upregulation of $\text{Ca}_v2.2$ [62].

Genetic ablation of $\text{Ca}_v2.2$ also confirmed its importance on chronic pain. Three groups independently generated $\text{Ca}_v2.2$ -/- mice and examined for pain behaviors [63-65]. Their results in acute pain are not consistent with each other. Kim *et al.* showed a reduction in mechanical acute pain but not thermal acute pain [64]. However, in two other groups, knockout mice only showed a reduction in thermal acute pain. In accordance with this inconsistency on acute pain, ω -conotoxins' effect on acute pain are conflicting [66-69]. However, in formalin test, all knockout mice showed delayed responses in phase II, suggesting N-type VGCCs are involved in inflammatory pain. Also, intrathecal ω -conotoxins potently block pain behaviors by neuropathic pain [45]. These results suggest antagonizing $\text{Ca}_v2.2$ is more beneficial in reducing pathological pain than acute pain, although the molecular basis of these differential effects remains elusive.

3.4. R-type Ca^{2+} Channels

HVA Ca^{2+} currents resistant to L-, P/Q-, and N-type VGCC blockers are R-type VGCC currents, which are generated by $\text{Ca}_v2.3$. The channel subtype is also expressed in DRG and the spinal cord. Like $\text{Ca}_v2.2$, there has been a report that $\text{Ca}_v2.3$ has several, at least six, isoforms. Among them, $\text{Ca}_v2.3(e)$ is expressed in small, IB4 negative, and capsaicin positive DRG cells, suggesting this isoform is potentially involved in pain signaling [70, 71]. In fact, SNX-482, an antagonist for R-type VGCCs, reduced hyperexcitability of C- and A δ - fibers in SNL neuropathic pain models [72, 73]. In terms of regulation, it has been shown that R-type VGCC currents was reduced by neuropathic pain models such as PSNL, while N-type VGCC currents was increased, suggesting adaptive changes into N-type VGCCs. However, interestingly, $\text{Ca}_v2.3$ -/- mice showed the adaptive changes into L-type VGCCs, suggesting a role of R-type VGCCs in adaptive mechanisms by neuropathic pain [74]. This might explain why $\text{Ca}_v2.3$ -/- mice showed normal behavior against acute pain [75, 76]. Also, there is a report that R-type VGCCs are expressed in PAG (periaqueductal gray) and has an anti-nociceptive role. These results show that, like P/Q-type VGCCs, the contribution of R-type VGCCs to pain is rather complicated.

3.5. T-type Ca^{2+} Channels

T-type VGCCs are broadly expressed in heart, muscle, brain, and peripheral nerve, suggesting its contribution to cardiac function, epileptics, sleep, and pain. In the pain signaling, T-type VGCCs ($\text{Ca}_v3.2$ and $\text{Ca}_v3.3$) also are expressed in small and medium DRG and spinal cord, being involved in excitability, neurotransmitter release, and pain sensitization. It has been reported that presynaptic $\text{Ca}_v3.2$ was involved in glutamate release in synapse of lamina I and II [77, 78]. In particular, spinal T-type VGCC currents was important in hyperalgesia in NK receptors positive lamina I neurons, emphasizing their role in central sensitization [79].

In terms of regulation, it has been shown that T-type VGCC currents are down-regulated by neuropathic pain condition such as the CCI model. Control mice showed 25% T-type VGCC current in medium sized DRG cells but these were abolished in the neuropathic pain model [80, 81]. However, more recently, it has been shown that T-type VGCC current is increased in small neurons of CCI neuropathy model and also that Ca_v3.2 and Ca_v3.3 mRNA is increased in rat chronic compression of DRG (CCD) model [82, 83]. The different regulations of T-type channels in small and medium sized DRG cells and their physiological effects should be further investigated. Possibly, medium sized DRG cells do not participate in pain circuitry. Shin *et al.* showed that Ca_v3.2 is involved in D-hair receptors. Ca_v3.2-/- mice showed no effects on mechanistic nociceptive C-fibers [84]. Despite the promiscuous role of T-type channels, other evidences indicated that Ca_v3.2 is involved in specific contexts of pain such as visceral pain and diabetic neuropathic pain. For example, butyrate, which is increased by irritable bowel syndrome, up-regulated T-type VGCC currents and antisense knockdown of Ca_v3.2 reversed the hypersensitivity [85]. Selective T-type VGCC blocker, (3β,5α,17β)-17-hydroxyestrane-3-carbonitrile, reduced thermal and mechanical allodynia from diabetic neuropathy in leptin deficient mice [86]. Silencing of Ca_v3.2 using antisense oligodeoxynucleotides also reduced hyperalgesia and allodynia of rat CCI model [87]. Further, antisense for Ca_v3.2 and Ca_v3.3 attenuated pain in CCD model [88]. In accordance with these knock down experiment, Ca_v3.2 knockout mice showed attenuation in pain behaviors [89]. Interestingly, there was a report that genetic ablation of Ca_v3.1 causes hypersensitivity to noxious visceral stimuli, which is involved in a lack of thalamic burst firing [90]. However, we cannot exclude the possibility of Ca_v 3.1 involvement in neuropathic pain such as the SNL model [91]. Taken together, among LVA VGCCs, peripheral Ca_v3.2 is an extremely prominent target for analgesic development.

3.6. α_{2δ} Subunits

α_{2δ} subunits increase Ca²⁺ current amplitude by increasing functional α₁ subunits at membrane site [92]. Therefore, α_{2δ} subunits have been used to increase Ca²⁺ currents in functional assay for VGCCs [93]. Also, these subunits modulate gating kinetics and pharmacology of α₁ subunits. These biophysical characteristics might affect hyperexcitability by neuropathic pain [94]. The importance of α_{2δ} subunits in chronic pain has been verified that the subunits are up-regulated by neuropathic and inflammatory pain [95]. For example, inflammation by intraplantar CFA increases mRNA and protein expression of α_{2δ} subunits [58]. It has been shown that mRNA levels of α_{2δ}1 are upregulated particularly in small DRG of PNSL models [96]. In addition, the relevance of α_{2δ} subunits in pain is supported by interesting evidence that α_{2δ} subunit homolog straightjacket is also responsible for thermal pain in *Drosophila* [97]. In accordance with this result, α_{2δ}3 mutant mice showed increased latency on thermal pain [97]. Recently, another mechanism of α_{2δ} has been revealed that the α_{2δ} subunit is responsible for synaptogenesis by interaction with thrombospondins [62]. This fact suggests

that the synaptogenesis by α_{2δ} subunit might affect hyperexcitability and chronic pain.

It has been known that gabapentinoids such as gabapentin and pregabalin alleviate neuropathic pain by reducing Ca²⁺ current. However, the direct inhibiting effects for VGCCs are controversial. Several report in the literature reported that gabapentin inhibited high-threshold Ca²⁺ current by acting on α_{2δ} subunits [98-100]. However, other evidences showed that these drugs did not show VGCC current blocking effects [92]. More likely, gabapentin may reduce excitability by inhibiting synaptogenesis. The exact mechanism of α_{2δ} in chronic pain remains elusive.

4. VGCC INHIBITORS

Three VGCC subtypes (Ca_v1.2, Ca_v2.2, and Ca_v3.2) play an important role in either DRG or the spinal cord in pain processing and chronic pain development. Given that these α₁ subunits are 24-multiple transmembrane domains, it is no wonder that various extracellular ligands (either exogenous or endogenous) and intracellular modulators interact with VGCCs and modulate functions of VGCC subtypes, thus resulting in regulation of chronic pain phenotypes. These regulators can be used for therapeutic developments for pathological pain. For example, ω-conotoxin MVIIA, which extracellularly interacts with N-type VGCCs, has been approved by U.S. FDA for refractory pain. A tat-associated peptide CRMP-2, which intracellularly interferes with the interaction between N-type VGCCs and collapsin (a synaptic protein enhancing the function of N-type Ca_v currents), showed analgesic effects [101]. These inhibitors were quite useful for pain therapeutic developments and strategy for targeting these into nociceptors.

4.1. L-type Ca²⁺ Channel Inhibitors

It has been well established that classic calcium channel blockers DHP, PAA, and BTZ mainly target L-type VGCCs. Nifedipine, verapamil, and diltiazem are representative calcium channel blockers, respectively (Fig. 2A) [102]. These has been called “Ca²⁺ antagonist” because they are neutralized with elevated Ca²⁺ concentration [103]. These drugs have been known to bind to pore forming regions (S5 or S6 in domain III and IV, Fig. 1). For example, isradipine (DHP) and devapamil (PAA) interact with S6 in domain IV [104]. Nitrendipine (DHP) binds to S5-S6 of domain III [105]. Diltiazem (BTZ) also binds to S5 and S6 in domain III and IV [106]. It has been known that the binding site of DHP is close to the extracellular surface based on the blocking effects of extracellular and intracellular application of charged DHP [107]. DTZ323, a quaternary 1,5-benzothiazepine analog, inhibited current extracellularly, but not intracellularly, showing that BTZ also interacts with the extracellular portion of Ca²⁺ channels. However, D890, quaternary form of methoxyverapamil, only inhibited Ca²⁺ current intracellularly, showing that binding site of PAA can be accessed inside of the cells. It should be noted that voltage-gated ion channels are moving targets which show conformational changes between closed, open, inactivated states. Some drugs showed different affinities against different state of channels. This phenomenon is called “state-dependency” of a drug, which results in “use dependency” or

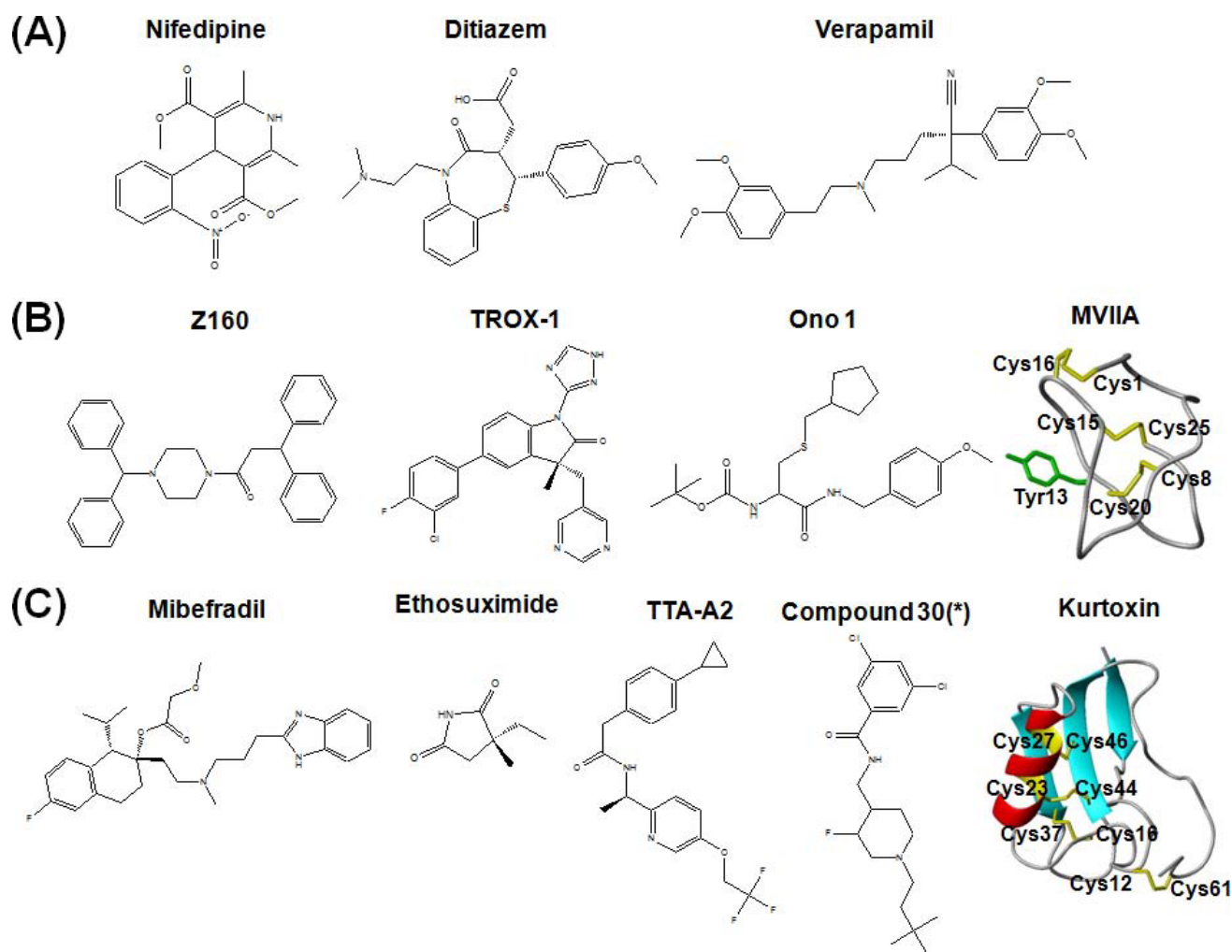


Fig. (2). Molecular structures of VGCC subtype blockers (A) L-type VGCC blockers (B) N-type VGCC blockers (C) T-type VGCC blockers. * indicates that Compound 30 is a label used in [168].

“frequency dependency” [108]. The state or use dependency of the drug is useful in terms of therapeutic developments because neuropathic pain is usually related to hyperexcitability. It has been shown that nitrendipine binds the inactivate state of cardiac calcium channels with subnanomolar affinity but the resting state with ~ 700 nM affinity [109]. Other than these small molecules, conotoxin TxVII blocks L-type VGCCs. Interestingly, TxVII inhibits DHP sensitive currents in cultured *Lymnaea* RPd1 neurons, but not in PC12 cells, suggesting that how it has subtype selectivity [110]. However, there is still no information which subtype is involved in the action of TxVII and it might modulate pain signaling.

As many authors mentioned, analgesic effects of L-type Ca_v blockers are ambiguous. Ca^{2+} channel blockers, including nifedipine, verapamil, and diltiazem had no effects on pain behaviors when injected intrathecally [111, 73]. However, intraperitoneal injection of verapamil reduced mechanical and thermal pain in rats. Also, intrathecal verapamil and flunarizine reduced thermal pain in an opioid receptor dependent manner [112, 113]. In the neuropathic pain model of SNL, nifedipine reduced pain behaviors [37].

It is probable that these blockers act on L-type VGCCs in both excitatory and inhibitory neurons in the spinal cord, showing sum effects of the blockers. Also, different affinities of blockers against $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ might explain the inconsistency of *in vivo* analgesic activities. Therefore, subtype specific blockers against $\text{Ca}_v1.2$ or the specific targeting of drugs into nociceptors might improve the analgesic effect of L-type VGCC blockers because of the preferential expression of $\text{Ca}_v1.2$ in nociceptor and its pain modulation.

4.2. N-type Ca^{2+} Channel Inhibitors

In contrast to the organic blockers for L-type VGCCs, peptide neurotoxins from cone snails have been used for isolation and characterization of N-type VGCCs. ω -conotoxins GVIA and MVIIA isolated from the venom of *Conus geographus* and *Conus magus*, respectively, block N-type VGCCs with nanomolar affinity (Fig. 2B). Electrophysiology using chimeric channels has shown that ω -conotoxin GVIA bind to S5 and H5 in domain III [114]. Binding sites of ω -conotoxins GVIA and MVIIA are partially overlapped, which suggest they have similar

binding mechanisms [115]. Due to their high specificity and nanomolar range affinity, they have been useful to characterize the function of N-type VGCCs in physiology. Eventually, synthetic ω -conotoxin MVIIA was approved by U.S. FDA in 2004 for refractory pain. Since confirming N-type VGCCs as a pain therapeutic target, many academics and companies have developed other peptide neurotoxins and active small molecules. The main reason of this is that the use of ziconotide has been limited due to uncomfortable intrathecal administration, severe side effects, and cost of production [116]. A good example is ω -conotoxin CVID isolated from *Conus catus*, which now is in clinical trials as Leconotide. This conotoxin has been improved in terms of selectivity for N-type over P/Q-type VGCCs. In behavioral tests, intrathecal ω -conotoxin CVID has a better therapeutic index in pain animal studies [69]. Surprisingly, a recent report showed that intravenously administered Leconotide has an analgesic effect without side effects [117]. This could circumvent the need for the uncomfortable intrathecal administration of Ziconotide. Another ω -conotoxin FVIIA, isolated from *Conus fulmen*, has a better reversibility compared to ω -conotoxin MVIIA, which might improve side effects and managements of ziconotide administration. Several spider toxins also showed improvements in terms of their effects on motor coordination. The examples are huwentoxin-I and PnTx3-6, which showed low motor deficits [118, 119]. However, all these peptide neurotoxins are still expensive to produce due to limited peptide synthesis and then low cysteine folding yield.

As for small molecules against $\text{Ca}_v2.2$, Takashi *et al.* described current developments of N-type VGCC blockers [120]. Several groups tried to develop small molecules based on a structural analysis of ω -conotoxin MVIIA or GVIA. Other groups tried to design and synthesize blockers based on pre-existing organic blockers. Currently, the developed small molecules have several moieties such as piperazine, 4-aminopiperidine, aryl sulfonamide, and aryl heterocycle moiety. The snutch group designed and synthesized two N-type VGCC blockers NP078585 and NP118809 to favor selectivity over L-type VGCCs. The chemical structures are based on a structure-activity relationship of flunarizine and lomerizine. NP118809 has been favored for further development due to low off-target activity to hERG K_v channels that mediate cardiac action potentials. Currently, it has been developed as Z160 (previously, NMED-160 or MK-6721) and is in phase IIa study by Zalicus (Fig. 2A). Methoxy groups adjacent to benzoyl linker of NP078585, which has trimethoxybenzyl piperazine moiety from flunarizine and lomerizine, are substituted with S-Me group, resulting in a high potency for N-type VGCCs [121, 122]. Merck designed and synthesized aminopiperidine sulfonamide inhibitors, which are state-dependent blockers. These compounds inhibit CFA-induced inflammatory pain and SNL neuropathic pain in a $\text{Ca}_v2.2$ expression-dependent manner [123]. TROX-1 was also discovered by Merck (Fig. 2A). An IC_{50} of this compound in a depolarized state is 400 nM in rat DRG, but in a hyperpolarized state is 2.6 μM , showing a characteristic of state dependent blockers. Oral 10mg/kg TROX-1 showed analgesic effects in inflammatory and neuropathic pain similarly with NSAIDs and pregabalin [124, 125]. Ono Pharmaceutical designed and synthesized L-

cysteine based N-type VGCC blockers (Fig. 2A). Oral administration of this compound (30mg/kg) showed analgesic effects in the rat formalin test and CCI neuropathic pain, but high dose (100mg/kg) did not have an effect on cardiac or motor function [126, 127].

4.3. T-type Ca^{2+} Channel Inhibitors

Mibefradil (Ro 40-5967), a tetralol derivative, was developed as an anti-anginal and anti-ischemic agent (Fig. 2C) [128, 129]. However, it was withdrawn by Roche due to its interaction with liver enzyme [130]. In spite of the withdrawal, it has been used for research due to its selectivity for T-type VGCCs. Intraperitoneal injection of mibefradil reduced mechanical and thermal pain [131]. In rat neuropathic pain model (SNL), intraplantar and intraperitoneal mibefradil showed dose dependent effects on mechanical and thermal allodynia, but intrathecal injection did not show any effect [132]. In contrast to this, intrathecal mibefradil decreased neuropathic pain by CCD models [83]. These data suggested the involvement of T-type VGCCs in acute and neuropathic pain. Interestingly, intraperitoneal mibefradil enhances antinociceptive effects of opioids and blocks its tolerance but not dependence, although its mechanism is not clear [133]. Intraperitoneal ethosuximide, another T-type VGCC blocker, showed lower efficacy than mibefradil [132]. Intrathecal ethosuximide also showed dose dependent inhibition of neuropathic pain by SNL and spinal hyperexcitability [134]. Intraperitoneal ethosuximide had analgesic effects on chemotherapy induced pain, which is resistant to opioids and MK801, an NMDA antagonist [135].

Inspired by analgesic effects with mibefradil and ethosuximide, many academic labs and companies have tried to find selective and potent T-type VGCC blockers [136]. One achievement is TTA-A2 synthesized by Merck (Fig. 2C). This compound blocked recombinant T-type VGCCs with an IC_{50} of 10~90 nM at more depolarized voltages (-75~-80mV, large fraction of channels in inactivated state), while with an IC_{50} of 4~22 μM at hyperpolarized voltages (-100~-110mV) in heterologous T-type VGCC expressed in HEK cells and native T-type VGCCs [137, 138]. This evidence showed use dependency of TTA-A2, which is useful for reducing hyperexcitability related to chronic pain [137, 138]. The analgesic effects of oral TTA-A2 were examined on acute pain and an irritable bowel syndrome model, which is butyrate-induced hypersensitivity. TTA-A2 inhibited both type of pain but did not reduce acute pain in $\text{Ca}_v3.2$ knockout mice. However, oral administration of TTA-A2 disturbs sleep in normal mice but not double knockout of $\text{Ca}_v3.1$ and $\text{Ca}_v3.3$, indicating possible side effects. TTA-A2 inhibited presynaptic T-type VGCCs in the superficial laminae of dorsal horn, only affecting excitatory synaptic transmission [77]. Merck also developed quinazolinone T-type VGCC blocker TTA-Q4, which showed synergetic effects with TTA-A2 [139]. A novel compound has been identified through high throughput screening to block $\text{Ca}_v3.1$ and $\text{Ca}_v3.3$ with 20~60 nM affinities. Their developments focused on epilepsy, but targeting this into the peripheral nervous system would be beneficial (Fig. 2C) [140, 141]. Recently, A803467, a blocker of TTX resistant $\text{Na}_v1.8$, has been examined on T-type VGCCs due to structural similarity of $\text{Na}_v1.8$ and Ca_v3 . It also inhibit T-type VGCCs with IC_{50}

of $\sim 5\mu\text{M}$ at a holding potential of -110mV and interestingly stabilized slow inactivated state of $\text{hCa}_v3.2$ (not $\text{Ca}_v3.1$ and $\text{Ca}_v3.3$) [142]. Therefore, analgesic mechanism of A803467 might partially result from T-type VGCC inhibition.

Zonisamide, 1,2-benzisoxazole-3-methanesulfonamide, is known as an anticonvulsant and inhibitor of repetitive firing, thus it is proposed as a T-type VGCC blocker [143]. It also was involved in central poststroke pain [144]. In a recent electrophysiological study, a high dose (2mM) of zonisamide partially inhibited $\text{Ca}_v3.2$ current with 30% block and did not show use-dependent block [145]. Trazodone, a treatment for insomnia and depression, also blocked heterogeneous T-type VGCC current with an IC_{50} of $23\sim 45\mu\text{M}$. Butamben, a local anesthetic, also inhibits T-type VGCC currents with an IC_{50} of $200\mu\text{M}$ in addition to N-type VGCCs. Interestingly, it accelerates T-type VGCC kinetics [146]. It has been shown that trimethadione, as a T-type VGCC blocker, inhibited hyperalgesia in a dose dependent manner. However, it does not inhibit LVA current in somatosensory thalamic neurons [147, 148].

It should be noted that many endogenous molecules modulate T-type VGCCs in addition to the small molecules described above. For example, it has been reported that the endogenous lipid NAGly (N-arachidonoyl glycine) or NAGABA-OH inhibited T-type VGCC currents with a preference to $\text{Ca}_v3.2$ by stabilizing inactivated state based on the observation of hyperpolarized shift in the steady-state inactivation [149]. These lipids blocked recombinant $\text{Ca}_v3.2$ with EC_{50} of $\sim 600\text{nM}$ and $\sim 200\text{nM}$, respectively, and showed selectivity over the cannabinoid receptor or TRPV1. In behavior tests using $\text{Ca}_v3.2$ -/- mice, these lipoamino acids showed thermal analgesia in a $\text{Ca}_v3.2$ -dependent manner. Other endogenous modulators of T-type VGCCs are 5β reduced neuroactive steroids [150, 151]. These endogenous molecules have potential to be developed into analgesics.

Another family of inhibitors modulating T-type VGCCs is peptides or protein neurotoxins produced by the venom organ of venomous animals such as spiders, scorpions, sea anemones, and cone snails. The founding member of T-type VGCC blocking toxin is kurtoxin, isolated from the venom of the scorpion *Parabuthus transvaalicus* [152]. It was reported that kurtoxin blocks $\text{Ca}_v3.1$. However, it turned out to block other HVA channels as well as $\text{Ca}_v3.1$ and $\text{Ca}_v3.3$ [153]. More recently, it has been reported that ProTx-I preferentially block $\text{Ca}_v3.1$ over $\text{Ca}_v3.2$ by a interaction with the putative voltage sensing domain (S3-S4 linker in domain IV) of $\text{hCa}_v3.1$ [154]. Three-dimensional structures of kurtoxin and ProTx-I have been determined by nuclear magnetic resonance. Ensuing structural studies will provide invaluable pharmacopore information similar to what was shown for the N-type VGCC blocker ziconotide [155].

5. PROSPECTS

VGCC subtype inhibitors including ziconotide (ω -conotoxin MVIIA) showed both potential and limitations as chronic pain therapeutics. Several strategies for overcoming the limitations have been implemented to enhance the inhibitor specificity for voltage-gated ion channels (either small molecule or peptide), some of which were already

applied to VGCC subtypes. These include silencing nociceptors, a development of use-dependent blockers, and expression of tethered peptide neurotoxins.

5.1. Silencing Nociceptor

Binshtok *et al.* reported an interesting approach to overcome low specificity of a local anesthetic lidocaine [156]. Although the strategy targeted VGSCs, it deserves consideration as a potential application to VGCCs. Lidocaine (pK_a 7.8) has more portions of lipid soluble forms, which can penetrate the cell membrane to reach its binding site. QX-314 (low pK_a), first synthesized by the Astra Pharmaceutical Company in the early 1970s, is a quaternary ammonium derivative of lidocaine. It is a permanently charged form and cannot permeate the membrane. Thus, it has been known to have a binding sidedness that only by internal application of QX-314 it reach its binding site. Binshtok *et al.* recognized that large molecule such as QX-314 can pass through the pore of activated TRPV1, which is expressed specifically in nociceptors. Therefore, nociceptor-specific internalization of QX-314 has been achieved through co-application of QX-314 and capsaicin, a ligand for TRPV1. Internalized QX-314 specifically blocked animal pain behaviors for several hours without any sensory or motor deficits. However, external QX-314 alone did not block sodium current. Internalization of QX-314 by capsaicin in the cells has been verified by tandem mass spectroscopy and electrophysiology [157, 158]. It is very meaningful that the specificity of VGSC blocker QX-314 is achieved by the localization of TRPV1, although previously QX-314 was only considered a research tool. In the same way, if small molecules for VGCCs have internal binding sidedness, this strategy should be considered. While targeting VGSCs result in inhibiting action potential generation for propagation of pain signaling, targeting VGCCs in nociceptors may be even more effective by blocking both synaptic transmission in presynaptic nerve terminals as well as peptide release in peripheral nerve ending.

There have been several studies that characterize the sidedness of permanently charged organic calcium antagonists. For example, the effect of charged DHP (amlodipine and SDZ 207-180) on L-type VGCC currents has been examined internally by electrophysiology. However, the internally applied charged DHP did not show any blocking effect, meaning DHP is not appropriate for this strategy [107, 159]. However, D-890 (N-methyl-verapamil) is active only when inside the cells so it might be a good option for this silencing strategy using TRPV1 channel [160]. It would be interesting if the recently discovered N-type and T-type VGCC blockers can be used in this strategy.

5.2. High Throughput Screening for Searching use Dependent Blockers

As described above, VGCCs are moving targets and thus several blockers can preferentially bind to the inactivated state; a common characteristics of hyperexcitable cells [161]. It has been suggested that ziconotide is a state-independent drug, which might influence side effects. Several academic and industry labs have tried to develop the use dependent

drugs for VGCCs. One way to discover use dependent drugs for VGCCs is *via* high throughput Ca²⁺ imaging experiments. Detection of intracellular Ca²⁺ influx using fluorescence dyes is a well-established readout of the function of VGCC subtypes [162]. Therefore, screening VGCC subtypes are relatively easy compared to other VGSC or VGPC subtypes. For high throughput screening, cell lines heterologously expressing VGCC subtypes is frequently used. However, typical cell lines (e.g. HEK cells) for screening have rather high membrane potentials. For example, HEK293 cell lines have a resting membrane potentials at -12mV, which inactivates most of the VGCC subtypes [163]. To overcome this, inward rectifier K⁺ channels are co-transfected with VGCC subtypes. After transfecting the K⁺ channels, the membrane potentials can be controlled with potassium concentrations for a use-dependent assay. One group used 4 mM and 14 mM KCl as baseline buffers, which represent resting and inactivated states of VGCC subtypes, respectively. They then used a high concentration of KCl (e.g. 80mM) to induce depolarization [125]. This high throughput assay was complemented by an automated patch clamp assay, which achieves accuracy before they conducted manual patch clamp [164]. Differently from HVA VGCC subtypes, screening for T-type VGCCs seems rather difficult because of the existence of a large portion of inactivated channels even at resting membrane potentials. Two groups already reported a T-type VGCC assay with co-transfection of T-type VGCCs and Kir2.2 [165, 166]. Another group used an interesting approach to use gramicidin to remove inactivation [167]. These improvements of methodologies for high throughput screening will help the identification of VGCC subtype blockers, some of which will show use-dependent activity.

5.3. Tethered Peptide Neurotoxin and Gene Therapy

Peptide neurotoxins are another large class of ion channel blockers. These toxins have a high proportion of cysteines in their sequences, which form multiple disulfide bonds and thus contribute to rigid structural integrity. These toxins have been isolated from the venom of cone snails, spiders, scorpions, snakes, and sea anemones. This means that peptide neurotoxins are encoded by genes and modifiable by genetic engineering. Interestingly, several endogenous cysteine-rich peptides or proteins also modulate voltage-gated ion channels. For example, lynx1 is a glycosylphosphatidylinositol (GPI) anchored protein which has 10 cysteines and modulates nicotinic acetylcholine receptors [168]. CKAMP-44 also has 8 cysteines and modulates AMPA receptors [169]. This protein has a transmembrane domain so that they are also membrane-tethered. Inspired by GPI anchored lynx1, Ibañez-Tallon *et al.* showed that peptide neurotoxins tethered by GPI has a blocking effect on $\alpha 7$ nicotinic acetylcholine receptor in Zebrafish, which could overcome for the problems of genetic ablation of ion channels such as compensation [170]. Tethered spider toxins targeting VGSCs are also expressed in *Drosophila* to affect circadian rhythm. Peptide neurotoxins blocking VGCC subtypes also have been tested in mice. Tethered fluorescent peptide neurotoxins, ω -conotoxin MVIIA and ω -Aga IVA inhibiting Ca_v2.2 and Ca_v2.1, respectively, have been

delivered through lenti virus to show a reduction in chronic pain [171].

Tethered peptide neurotoxins can be used for two purposes. One is for novel peptide neurotoxin screenings to identify their function. A large set of peptide neurotoxin genes have been characterized without identification of their respective functions. It is difficult to characterize their functions one by one through peptide neurotoxin synthesis and functional assay. It is relatively easier to use model animals such as *Drosophila* and *C. elegans*, which have well documented behaviors. This approach will accelerate identifying peptide blockers for VGCC subtypes. The other purpose is to use tethered peptide neurotoxins for gene therapy. Potentially, the tethered ω -conotoxin MVIIA can be targeted into specific cells (eventually nociceptors) by virus targeting [172]. Although safety concerns still remain to be considered, as gene therapy has only recently been approved in Europe, gene therapy using tethered peptides can be a promising way to treat neuropathic pain [173].

6. CONCLUSION

Pain therapeutics for chronic pain still have unmet need because current therapy is unsatisfactory mainly due to low efficacy or high side effects. Considering the important roles of VGCC subtypes in neurotransmitter release, excitability, and neurogenic inflammation, VGCC subtypes (Ca_v1.2, Ca_v2.2, Ca_v3.2, and $\alpha 2\delta$) in nociceptors are promising targets for chronic pain. Many VGCC subtype blockers that have been developed have contributed to chronic pain therapeutics and understanding the role of VGCC subtypes. Known and novel VGCC blockers (or identified by improved high throughput screening methods) can be targeted into nociceptor improve medication for chronic pain in terms of high efficacy and safety.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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